

# *Cordyceps sinensis* Acts as an Adenosine A<sub>3</sub> Receptor Agonist on Mouse Melanoma and Lung Carcinoma Cells, and Human Fibrosarcoma and Colon Carcinoma Cells

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## ABSTRACT

*Cordyceps sinensis*, a parasitic fungus on the larva of *Lepidoptera*, has been used as a traditional Chinese medicine. We previously reported that the growth of B16-BL6 mouse melanoma (B16-BL6) cells and mouse Lewis lung carcinoma (LLC) cells was inhibited by cordycepin (3'-deoxyadenosine), an ingredient of *Cordyceps sinensis*, and its effect was antagonized by MRS1191, a selective adenosine A<sub>3</sub> receptor (A<sub>3</sub>-R) antagonist although adenosine (up to 100 μM) had no effect on the growth of B16-BL6 and LLC cells. In this study, we investigated whether water extracts of *Cordyceps sinensis* (WECS) inhibit the growth of B16-BL6 cells, LLC cells, HT1080 human fibrosarcoma (HT1080) cells and CW-2 human colon carcinoma (CW-2) cells via their A<sub>3</sub>-R. As a result, the growth of all cell lines were potently inhibited by WECS (10 μg/mL) and the inhibitory effect of WECS was significantly antagonized by MRS1191 (1 μM). Furthermore, WECS included 2.34% w/w cordycepin and 0.12% w/w adenosine as components according to the HPLC-ECD system. In conclusion, WECS inhibited the proliferation of four cancer cell lines by stimulation of A<sub>3</sub>-R and the main component in WECS with anticancer action might be cordycepin instead of adenosine.

**Keywords:** *Cordyceps sinensis*, Adenosine A<sub>3</sub> Receptor, Cordycepin, HPLC-ECD

## 1. Introduction

*Cordyceps sinensis*, a fungus parasitized on the larva of *Lepidoptera*, has been used as a valued traditional Chinese medication and a tonic food. Natural products of *Cordyceps sinensis* are so rare and difficult to obtain in uniform composition that cultured products have been developed. In 1987, CordyMax Cs-4, a mycelial fermentation product of *Cordyceps sinensis*, was approved by the National New Drug Review and Approval committee of the Chinese Ministry of Public Health, and has been used in clinics for fatigue, night sweating, male and female hyposexuality including impotence, hyperglycemia, hyperlipidemia, asthenia after severe illness, respiratory diseases, renal dysfunction and renal failure, arrhythmias and other heart diseases, and liver diseases [1]. However, we focused on the cultural fruiting body of *Cordyceps sinensis* instead of the mycelium and reported the anti-

cancer effect of water extracts of *Cordyceps sinensis* (WECS) on mouse Lewis lung carcinoma (LLC) cells and B16 mouse melanoma cells [2].

In this study, we tried to elucidate the mechanism of the anticancer effect of WECS and speculated an active ingredient in WECS. In particular, we investigated pharmacologically whether WECS stimulate adenosine A<sub>3</sub> receptor (A<sub>3</sub>-R) using a selective A<sub>3</sub>-R antagonist since an effective component in WECS, cordycepin (3'-deoxyadenosine), showed anticancer action via the stimulation of A<sub>3</sub>-R, according to our previous experimental data [3]. Finally, we quantified the content of cordycepin in WECS using the HPLC-ECD system.

## 2. Materials and Methods

### 2.1. Chemicals

Dried fruiting bodies of cultured *Cordyceps sinensis* were

produced by Jiang Men Baode Biological Technology (Guangdong, China). They were extracted with hot water (70°C) for 5 min and the extract was then filtered and lyophilized. The lyophilized powder (WECS) was sealed in bottles and kept in a refrigerator (4°C) until use. 3-Ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-di-carboxylate (MRS1191, a selective A<sub>3</sub>-R antagonist), cordycepin (3'-deoxyadenosine), adenosine and fetal bovine serum (FBS) were purchased from Sigma Chemical Co. (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) with L-glutamine, Earle's minimum essential medium (EMEM) plus glutamax, RPMI1640 medium and MEM non-essential amino acids (NEAA) 100 × solution were from Invitrogen Co. (Grand Island, NY). Dulbecco's phosphate-buffered saline without calcium and magnesium [DPBS (-)] was from Nissui Pharmaceutical Co., Ltd (Tokyo, Japan). EDTA trypsin solution (EDTA, 0.02%; trypsin, 0.1%) and penicillin/streptomycin solution (penicillin, 50,000 U/mL; streptomycin, 50 mg/mL) were obtained from Cosmo Bio Co., Ltd. (Tokyo, Japan).

## 2.2. Cells

The mouse B16-BL6 epithelial-like melanoma cell line was kindly provided by Professor Futoshi Okada of Tottori University (Yonago, Japan). The mouse Lewis lung carcinoma cell line and CW-2 human colon carcinoma cell line were provided by the RIKEN CELL BANK (Tsukuba, Japan). The human HT1080 epithelial-like fibrosarcoma cell line was purchased from DS Pharma Biomedical Co., Ltd. (Suita, Japan). B16-BL6 cells were cultured in DMEM containing 10% FBS and a 0.1% penicillin/streptomycin solution. LLC cells were cultured in DMEM containing 10% FBS. HT1080 cells were cultured in EMEM containing 10% FBS and a 1% NEAA. CW-2 cells were cultured in RPMI1640 containing 10% FBS.

## 2.3. Growth Curves of B16-BL6 Cells, LLC Cells, HT1080 Cells and CW-2 Cells

Sub-confluent cancer cells were harvested with EDTA trypsin solution and resuspended as  $1 \times 10^5$  cells/2 mL in each well of a 12-well culture plate, and then treated with 10 µg/mL WECS. MRS1191 (0.1 and 1 µM) was added to the wells 30 min before the addition of WECS. To evaluate the effect of MRS1191 alone on cell growth, 0, 0.1, 1 and 10 µM of MRS1191 were added to each culture plate at 0 h. After 24, 48 and 72 h incubation at 37°C, viable cells were counted with a Coulter counter (Coulter Z1; Beckman Coulter, Inc., Tokyo, Japan).

## 2.4. Determination of Cordycepin and Adenosine Content in WECS

HPLC with electrochemical detection (HPLC-ECD) was used to quantify cordycepin and adenosine in WECS, according to a previously described method [4]. Briefly, 20 µL of WECS (1 mg/mL) was injected into the HPLC-ECD system. The analytical column was a Cosmosil 5C18-AR-II (particle diameter, 5 µm; column size, 150 × 4.6 mm I.D.; Nacalai Tesque, Kyoto, Japan) column equipped with a guard column (10 × 4.6 mm I.D.) and the mobile phase was 20 mM sodium phosphate buffer, pH 7.0, including 10% (v/v) methanol. The flow rate was 1 mL/min and the column temperature was set at 25°C. Quantitation of cordycepin and adenosine was performed by comparison of the peak area with those of authentic cordycepin and adenosine, respectively.

## 2.5. Statistical Analyses

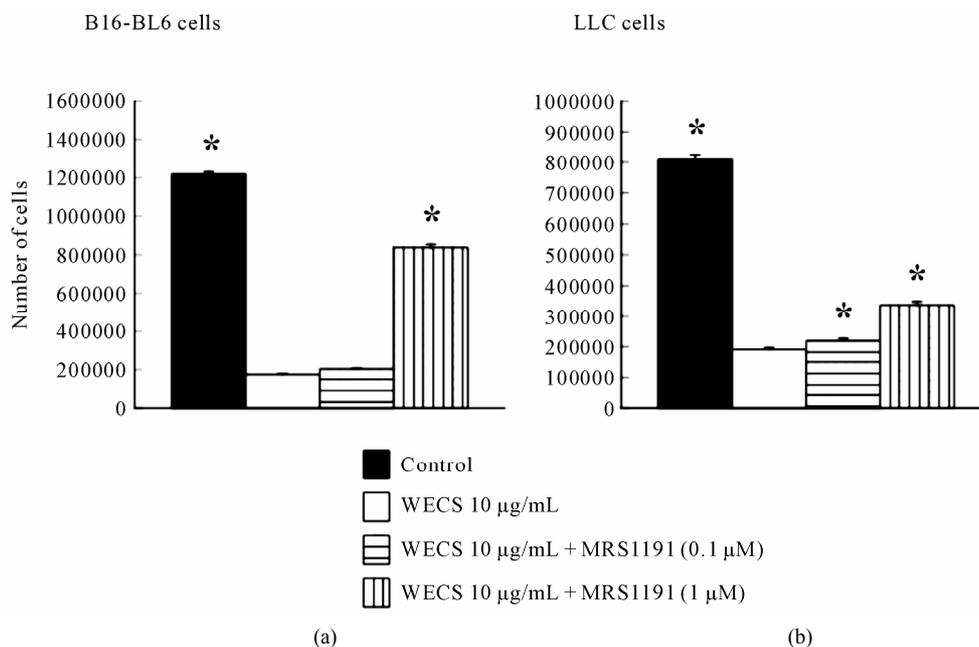
The data are expressed as the mean ± S.D. (N = 3). Statistical analyses were performed by one-way ANOVA followed by Tukey's Multiple Comparison Test using PRISM Version 4 (GraphPad Software, Inc., San Diego, CA). Differences were considered significant at  $P < 0.05$ .

## 3. Results

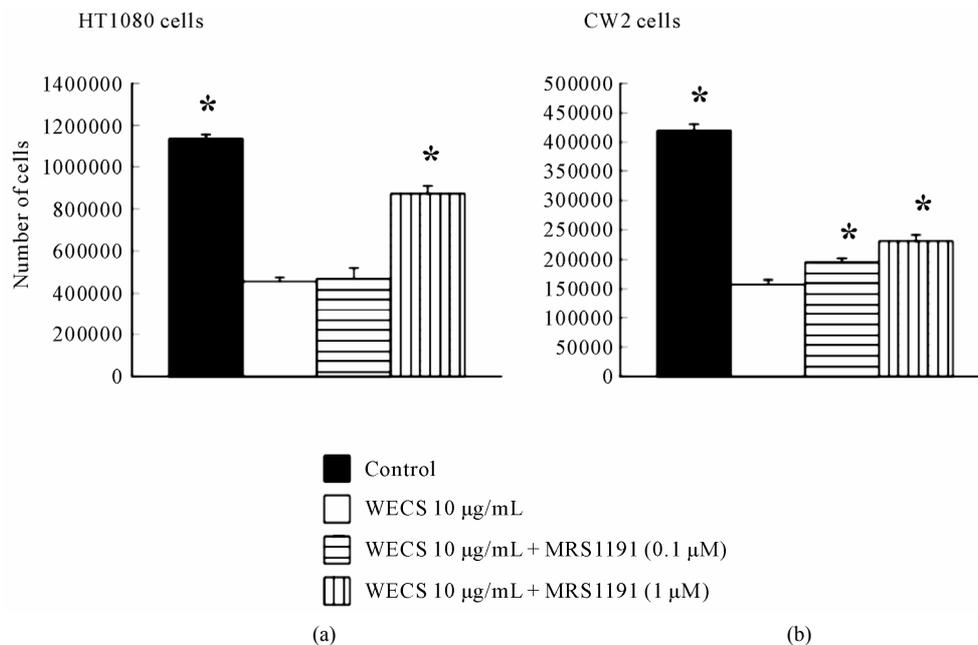
In mouse cancer cell lines, WECS (10 µg/mL) inhibited the growth of B16-BL6 cells and LLC cells potently to 14% and 23% of the non-treated control at 72 h, respectively. When B16-BL6 cells were premixed with 1 µM MRS1191, a selective A<sub>3</sub>-R antagonist, 30 min before the addition of WECS (10 µg/mL), the cell numbers were significantly antagonized to 69% of the control value. When LLC cells were premixed with 0.1 and 1 µM MRS1191, 30 min before the addition of WECS (10 µg/mL), the cell numbers were significantly antagonized to 27% and 41% of the control value, respectively (**Figure 1**).

In human cell lines, WECS (10 µg/mL) inhibited the growth of HT1080 cells and CW-2 cells potently to 40% and 38% of the non-treated control at 72 h, respectively. When HT1080 cells were premixed with 1 µM MRS1191, 30 min before the addition of WECS (10 µg/mL), the cell numbers were significantly antagonized to 77% of the control value. When CW-2 cells were premixed with 0.1 and 1 µM MRS1191, 30 min before the addition of WECS (10 µg/mL), the cell numbers were significantly antagonized to 46% and 55% of the control values, respectively (**Figure 2**).

In **Figure 3**, showing representative data using CW-2 cells, 10 µM MRS1191 slightly reduced the cell number; however, 0.1 and 1 µM MRS1191 alone did not have any



**Figure 1.** Effects of MRS1191, a selective A<sub>3</sub>-R antagonist, on mouse B16-BL6 (a) and LLC (b) cell growth inhibited by WECS. At time 0,  $1 \times 10^5$  sub-confluent B16-BL6 (a) and LLC (b) cells in 2 mL medium per well, obtained as a monodispersed suspension by trypsinization, were seeded into a 12-well culture plate in the presence of 10 µg/mL WECS. At 72 h after plating, cultures were trypsinized and viable cells of samples were counted using a Coulter counter. MRS1191 (0.1 and 1 µM) was added 30 min before the addition of WECS. Each bar represents the mean  $\pm$  S.D. (N = 3). \*P < 0.05 vs WECS 10 µg/mL.



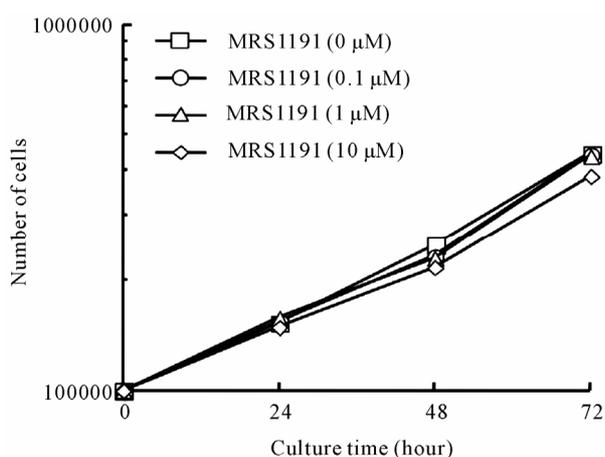
**Figure 2.** Effects of MRS1191, a selective A<sub>3</sub>-R antagonist, on human HT1080 cell (a) and CW-2 cell (b) growth inhibited by WECS. At time 0,  $1 \times 10^5$  sub-confluent HT1080 (a) and CW-2 (b) cells in 2 mL medium per well, obtained as a monodispersed suspension by trypsinization, were seeded into a 12-well culture plate in the presence of 10 µg/mL WECS. At 72 h after plating, cultures were trypsinized and viable cells of samples were counted using a Coulter counter. MRS1191 (0.1 and 1 µM) was added 30 min before the addition of WECS. Each bar represents the mean  $\pm$  S.D. (N = 3). \*P < 0.05 vs WECS 10 µg/mL.

effect on cancer cell growth at 72 h.

As shown in **Table 1**, the content of cordycepin and adenosine in WECS were 2.34% w/w and 0.12% w/w, respectively. WECS included cordycepin more abundant than adenosine.

#### 4. Discussion

To date, we have reported that WECS inhibited the growth of B16 melanoma cells and LLC cells [2] and cordycepin (3'-deoxyadenosine), an effective ingredient in WECS, also showed anticancer effects using B16-BL6 cells and LLC cells by stimulating A<sub>3</sub>-R [3]. As an effective ingredient, in addition to cordycepin in WECS, Chen *et al.* demonstrated that polysaccharide in cultivated *Cordyceps sinensis* significantly inhibited hepatoma H22 tumor cell growth [5]. In fact, polysaccharide is a potent candidate in WECS showing anticancer action; however, it is still a crude ingredient and its exact structure and molecular weight cannot be determined. WECS is also a crude product and its quality needs to be checked for commercial use. In other words, polysaccharide cannot



**Figure 3.** Effects of MRS1191, a selective A<sub>3</sub>-R antagonist on human CW-2 cell growth. At time 0,  $1 \times 10^5$  sub-confluent CW-2 cells in 2 mL medium per well, obtained as a monodispersed suspension by trypsinization, were seeded into a 12-well culture plate in the presence of 0, 0.1, 1 and 10 μM MRS1191. At 24, 48 and 72 h after plating, cultures were trypsinized and viable cells of samples were counted using a Coulter counter. Data represent the mean of triplicate samples.

**Table 1.** The content of cordycepin and adenosine in WECS.

Components	nmole/mg WECS	% w/w
Cordycepin	93.1 ± 0.3	2.34 ± 0.01
Adenosine	4.6 ± 1.3	0.12 ± 0.04

Data represents the mean ± S.D. of three independent experiments.

be utilized as a marker of the quality of WECS; however, cordycepin is a potent target candidate in quality checks because cordycepin was isolated as a pure compound in WECS and showed anticancer effects, and its structure and molecular weight were clarified (251.24). Accordingly, we considered cordycepin as an active ingredient showing anticancer action in WECS and tried to investigate whether the anticancer action of WECS was inhibited by A<sub>3</sub>-R antagonist, just like cordycepin. As a result, the anticancer effect of WECS was significantly antagonized by MRS1191, a selective A<sub>3</sub>-R antagonist; that is to say, cordycepin is a potent ingredient in WECS showing anticancer effects and can also be utilized as a marker for testing the quality of WECS. Actually, we determined the content of cordycepin as 2.34% w/w in WECS using the HPLC-ECD system in this experiment and it is much higher than that of adenosine (0.12% w/w in WECS). Although it is apparent that cordycepin might be a component showing anticancer action in WECS, other components also contribute to the anticancer action of WECS. Because the inhibitory effects of WECS on HT1080, B16-BL6, CW-2 and LLC cells were incompletely recovered to 77%, 69%, 55% and 41% of non-treated control values by addition of the selective A<sub>3</sub>-R antagonist, respectively, it is conceivable that the different responsibilities among the four cell lines probably occurred based on the variation in level of expression of A<sub>3</sub>-R on cell line membrane. We have already shown that B16-BL6 cells express A<sub>3</sub>-R according to a radioligand binding assay using [<sup>125</sup>I]-AB-MECA (a selective A<sub>3</sub>-R agonist) [6] and we speculate that HT1080 cells express a higher level of A<sub>3</sub>-R on the cell membrane than LLC, CW-2 and B16-BL6 cells due to their greatest responsibility to the selective A<sub>3</sub>-R antagonist.

#### 5. Conclusions

We clarified that the growth inhibitory effect of WECS on B16-BL6, LLC, HT1080 and CW-2 cells was significantly antagonized by the selective A<sub>3</sub>-R antagonist.

It is reasonable that the active ingredient in WECS indicating anticancer action might be cordycepin, which in WECS should be the target marker for testing the quality of WECS for commercial use.

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